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# Poliovirus Replicase Stimulation by Terminal Uridylyl Transferase*

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**In an *in vitro* poliovirus replication system, purified viral polymerase, plus sense virion RNA, and a host factor have been previously shown to be necessary for the transcription of minus strands. We have found that a partially purified eukaryotic initiation factor-2 (eIF-2) fraction from rabbit reticulocytes can replace HeLa host factor in the replicase reaction. This enzyme preparation contains eIF-2 and two other major proteins. In addition to eIF-2 activity, which does not appear to play a role in the replicase reaction, we find that the fraction contains terminal uridylyl transferase activity. The enzyme adds UMP moieties to the 3′ end of primer RNA molecules. The number of UMP residues added depends on the primer. Although long tails of heterogeneous lengths (50 to 100 nucleotides) can be polymerized on the 3′ end of oligo(U), a poly(A) primer accepts only four U's. The terminal uridylyl transferase activity requires only UTP, $Mg^{2+}$, a sulfhydryl reagent, and an RNA primer for activity. It is partially associated with ribosomes. We provide preliminary evidence that it may be responsible for host factor-like activity. We present a model for minus strand synthesis by poliovirus replicase, based on the hypothesis that a terminal uridylyl transferase can participate in initiation.**

Poliovirus has a 35 S RNA genome of positive polarity. The 5′-terminal nucleotide is covalently linked to a protein (VPg) and the 3′ end consists of a heterogeneous poly(A) tract averaging 75 nucleotides (1–6). The first step in replication must be the copying of a negative strand from the infecting positive strand which can then serve as template for the amplification of positive sense virion and messenger RNA sequences (7, 8).

Poliovirus replication can be studied using a soluble *in vitro* system which is believed to model the synthesis of the negative strand (9–16). Two protein fractions have been identified which are essential for reconstitution of transcription of virion RNA (9–14). One of these contains the RNA-dependent RNA polymerase encoded by the virus (p63) (13). This enzyme can be assayed as a poly(A)·oligo(U)-dependent poly(U) polymerase (17). The polymerase apparently requires a primer for initiation but no accessory factors for elongation (14–16). Only one molecule, oligo(U), has been proven to act as a primer for the copying of poliovirus RNA in the replicase reaction. Investigators have speculated that the VPg found covalently linked to the 5′ end of both virion RNA and negative strand copies may participate in initiation (18–22) but this has never been established. The second required protein, "host factor," obviates the need for an exogenous primer in the *in vitro* replicase reaction (9, 10, 12). Host factor was isolated from HeLa cells by virtue of its ability to restore activity to purified viral polymerase. It is probably a 67,000 molecular weight cytoplasmic protein (10, 12). Because the polymerase is competent for elongation, host factor is believed to correct a defect in initiation. The mechanism of initiation by host factor has not been determined. No polymerase activity has been detected in host factor preparations and there is no evidence that host factor can interact with VPg.

VPg is found linked to the 5′ end of both positive and negative strands (1, 3, 20). VPg-pUpU has been purified from infected cells and generated in crude *in vitro* membrane systems (21, 22). Nevertheless, there is no evidence that labeled VPg in any form can be incorporated into newly made polio viral RNA in the *in vitro* systems. [$^{32}$P]UTP-labeled products of the *in vitro* replicase reaction can be immunoprecipitated by anti-VPg antibodies (18, 23) but they are small heteropolymers which may be unrelated to replication. Anti-VPg antibody inhibits *in vitro* replication (18, 19) but this effect has not been pursued. Because direct attempts to find VPg in active polymerase preparations have failed (55) new approaches to understanding *in vitro* replication have been sought by focusing on possible activities of the host factor.

In this report, we describe co-purification of host factor activity with initiation factor eIF-2[1] through many purification steps. We tested protein synthesis initiation factor preparations for stimulation of the *in vitro* replicase reaction and found that a protein in a slightly contaminated eIF-2 fraction, but not pure eIF-2, could replace host factor. We provide evidence that the eIF-2-associated, host factor-like activity is a terminal uridylyl transferase. Similar enzymes have been described previously (24–30). Zabel and co-workers have described a plant enzyme with many of the same properties (30). The simplest explanation for the role of a terminal uridylyl transferase in the *in vitro* replicase reaction is that it puts an oligo(U) tail onto the 3′-terminal poly(A) of polio RNA, which can then fold back to form a hairpin. This type of structure might serve as a template/primer for the viral RNA polymerase.

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[1] The abbreviations used are: eIF-2, eukaryotic initiation factor-2; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

## EXPERIMENTAL PROCEDURES[2]

## RESULTS

*Host Factor Activity in eIF-2 Preparations*—Previously we had noted that ribosomal salt wash preparations contain a factor that acts as host factor in stimulating the poliovirus replicase reaction (9). We could repeat this observation using eIF-2 preparations purified from salt-washed rabbit reticulocyte ribosomes. The purification of eIF-2 is described under "Experimental Procedures." It involved ammonium sulfate precipitation of salt wash proteins, chromatography on DEAE and phosphocellulose columns, and finally sedimentation through a glycerol gradient. The peak glycerol gradient fractions yielded very highly, but incompletely purified eIF-2 (designated step VI eIF-2). This material (and material from earlier steps in the purification[3]) stimulated [$\alpha$-$^{32}$P]AMP incorporation in the *in vitro* polio replicase reaction (Table I). Step VI-N eIF-2, a greater than 95% homogeneous preparation (see "Experimental Procedures" for a description of its preparation), did not stimulate incorporation of AMP by replicase. Step VI eIF-2 did not incorporate [$\alpha$-$^{32}$P]AMP in the absence of viral polymerase. Two polymerase preparations, one completely host factor-dependent and the other partially host factor-dependent, were both stimulated. Further experiments were designed to characterize the activity of the factor in the step VI eIF-2 fraction.

The step VI eIF-2 fraction which can substitute for host factor contains the $\alpha$, $\beta$, and $\gamma$ subunits of eIF-2, plus two additional major protein species of 60,000 and 95,000 molecular weight (Fig. 1).

*Step VI eIF-2 Contains Terminal Uridylyl Transferase Activity*—[$\alpha$-$^{32}$P]UMP, like [$\alpha$-$^{32}$P]AMP, could be incorporated in the replicase reaction stimulated by step VI eIF-2. Incorporation of UMP, unlike AMP, was also stimulated in the absence of added viral polymerase (Table II). UMP incorporation by the eIF-2 fraction alone required RNA, but, as will become evident, polio RNA could be replaced by other RNAs, such as oligo(U). Thus it appeared that the host factor might have a terminal uridylyl transferase activity of the sort described previously (24–30).

The potential uridylyl transferase activity in step VI eIF-2 was studied further in a reaction using oligo(U) as a primer and lacking any poliovirus components (Table III). The reaction required the primer, a sulfhydryl reagent, and $Mg^{2+}$. Step VI-N eIF-2 did not contain this activity. It was inhibited by salt and aurintricarboxylic acid. When each of the four ribonucleotides was used individually as substrate, UTP was by far the best (Table IV). Of the others, only CTP showed definite incorporation. The incorporation of UMP was linear for at least 45 min, and continued for almost 2 h (Fig. 2*A*). About 2 $\mu$M oligo(U) primer was saturating (Fig. 2*B*).

To examine whether the primer was elongated during the reaction, a 5′ $^{32}$P-labeled, 19-nucleotide long oligo(U) fraction (with a trace of 18-mer) was isolated. It was incubated with unlabeled UTP and step VI eIF-2 and analyzed by electrophoretic separation (Fig. 3). With time, labeled RNA molecules increased in size by increments of 1 residue, indicating that the primer was extended during the reaction.

To further examine the primer-product linkage, we relied on the specificity of RNase $T_2$ to perform a nearest neighbor analysis. RNase $T_2$ cleaves all phosphodiester bonds in RNA to leave 3′ mononucleotides. RNA products of the terminal uridylyl transferase reaction using various primers were digested with RNase $T_2$ and chromatographed on thin layer cellulose plates with an isobutyric acid/$NH_4OH$/$H_2O$ solvent system. RNase $P_1$ digests were performed as a control, and showed that all labeled phosphate groups were still in the $\alpha$ position in uridyl nucleotides.[3]

TABLE I

*Step VI eIF-2 can replace host factor in the in vitro poliovirus replicase reaction*

Replicase reactions were performed as described under "Experimental Procedures" using partially pure polymerase (purification protocol 1) or pure polymerase (protocol 2). Incorporation of input [$\alpha$-$^{32}$P]ATP was measured by precipitation with trichloroacetic acid. Preparation of step VI and step VI-N eIF-2 is also described under "Experimental Procedures." In this experiment, about 0.4 $\mu$g of protein from step VI eIF-2 was used, and about 2 $\mu$g of protein from step VI-N.

| Conditions | *pmol [$^{32}$P]AMP incorporated* |
|---|---|
| Experiment 1 (pure polymerase) | |
| Complete | 0.3 |
| Minus polymerase, minus step VI eIF-2 | <0.02 |
| Minus polymerase | <0.02 |
| Minus step VI eIF-2 | <0.02 |
| Minus step VI eIF-2, plus step VI-N eIF-2 | 0.02 |
| Experiment 2 (partially pure polymerase) | |
| Complete | 2.5 |
| Minus polymerase, minus step VI eIF-2 | <0.01 |
| Minus polymerase | <0.01 |
| Minus step VI eIF-2 | 0.9 |
| Minus stev VI eIF-2, plus step VI-N eIF-2 | 1.0 |

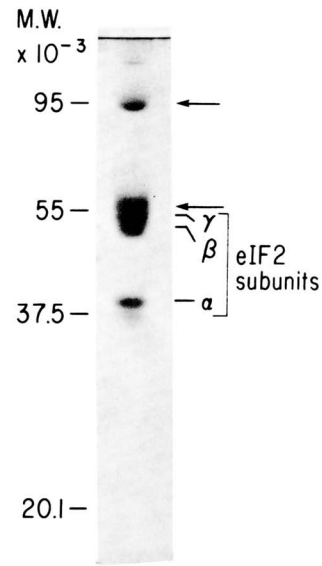


FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel showing protein components of step VI eIF-2.** Step VI eIF-2 was electrophoresed through a 10% protein gel, and the gel was silver stained. The three subunits of eIF-2 are indicated, migrating at about 38,000, 50,000, and 52,000 molecular weights. *Arrows* indicate two additional major protein bands, at approximately 60,000 and 95,000. There is one minor band visible at high molecular weight. The gel was overloaded to show any minor bands which might have been present.

[2] Portions of this paper (including "Experimental Procedures" and Footnote 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3846, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[3] N. C. Andrews, unpublished results.

TABLE II

*Incorporation of [α-$^{32}$P]UTP in the replicase reaction stimulated by step VI eIF2*

Replicase reactions using [α-$^{32}$P]UTP were performed as described under "Experimental Procedures" using enzyme fractions also described in that section.

| Conditions | *pmol [$^{32}$P]UMP incorporated* |
|---|---|
| Experiment 1 (pure polymerase) | |
| Complete | 0.93 |
| Minus polymerase, minus step VI eIF-2 | <0.03 |
| Minus polymerase | 0.09 |
| Minus step VI eIF-2 | <0.03 |
| Experiment 2 (partially pure polymerase) | |
| Complete | 1.6 |
| Minus polymerase, minus step VI eIF-2 | <0.03 |
| Minus polymerase | 0.09 |
| Minus step VI eIF-2 | 0.04 |
| Experiment 3 (partially pure polymerase) | |
| Complete | 1.1 |
| Minus RNA, minus polymerase, minus step VI eIF-2 | <0.004 |
| Minus RNA, minus polymerase | <0.004 |
| Minus RNA, minus step VI eIF-2 | <0.004 |
| Minus RNA | <0.004 |

TABLE III

*Biochemical characteristics of the UMP-incorporating activity present in step VI eIF-2*

Biochemical characteristics of UMP incorporation by step VI eIF-2 were investigated using the terminal uridylyl transferase assay described under "Experimental Procedures."

| Conditions | [$^{32}$P]UMP incorporated |
|---|---|
| | *% of complete* |
| Complete | (100) |
| Minus dithiothreitol | 33 |
| Minus oligo(U) | 7 |
| Minus enzyme | <1 |
| Minus enzyme, plus step VI-N eIF-2 | <1 |
| Minus $Mg^{2+}$ | <1 |
| Plus 40 mM KCl | 30 |
| Plus 100 mM KCl | 15 |
| Plus 10 μg/ml actinomycin D | 125 |
| Plus 0.1 μM aurintricarboxylic acid | 18 |
| Plus 10 μM aurintricarboxylic acid | <1 |

TABLE IV

*Incorporation of different NTPs by step VI eIF-2*

Each of the 4 NTPs was substituted for UTP as the sole nucleotide in terminal uridylyl transferase reactions as described under "Experimental Procedures."

| [α-$^{32}$P]NTP added | pmol incorporated | % of UMP |
|---|---|---|
| UTP | 0.52 | (100) |
| ATP | 0.01 | 2 |
| CTP | 0.08 | 15 |
| GTP | 0.01 | 2 |

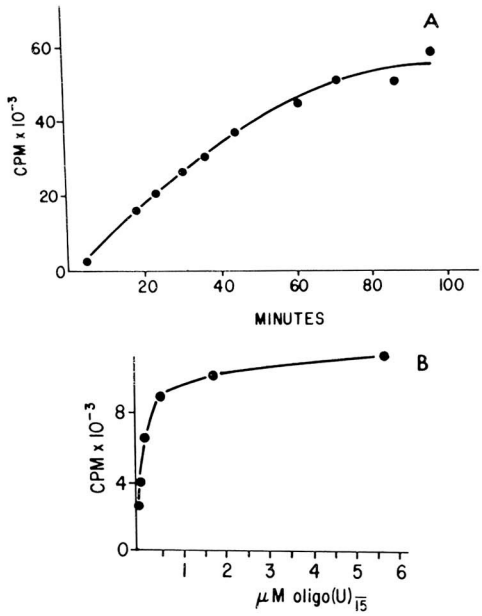


FIG. 2. **Kinetic behavior of uridylyl transferase.** *A*, incorporation of UMP by step VI eIF-2. A terminal uridylyl transferase assay (see "Experimental Procedures") of 10 times the standard volume was incubated at 30 °C for 2 h, and samples were withdrawn at various time points to measure incorporation. *B*, substrate titration of step VI eIF-2 incorporation of UMP. Step VI eIF-2 protein was added to terminal uridylyl transferase reactions with varying concentrations of oligo(U) substrate (see "Experimental Procedures"). The molar concentration of oligo(U) was estimated assuming that the average size of molecules in the preparation was 15 nucleotides in length (this is reasonable based on electrophoretic analysis of the oligo(U) preparation[3]).

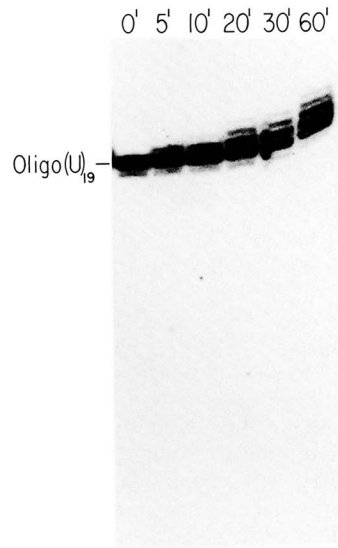


FIG. 3. **Extension of 5′-labeled oligo(U).** Terminal uridylyl transferase reactions were performed in which the $^{32}$P label was at the 5′ end of oligo(U), rather than in UTP. The preparation of 5′ end-labeled oligo(U)$_{19}$ is described under "Experimental Procedures." The oligo(U)$_{19}$ used in this experiment was contaminated with a small amount of oligo(U)$_{18}$. Samples were removed at various time points and analyzed by electrophoresis through urea/polyacrylamide gels as described under "Experimental Procedures."

Nearest neighbor analysis of the products formed using synthetic RNA homo- or heteropolymers as primers showed that a short tail of U's had been added to the 3′ end of the primer molecules (Fig. 4). There were spots corresponding to nucleotides of the input RNA and to Up (3′-UMP), indicating that α-phosphate groups from [α-$^{32}$P]UTP could label the 3′ end of the primer RNA and also other uridyl residues which had been added by the uridylyl transferase. All of the RNA

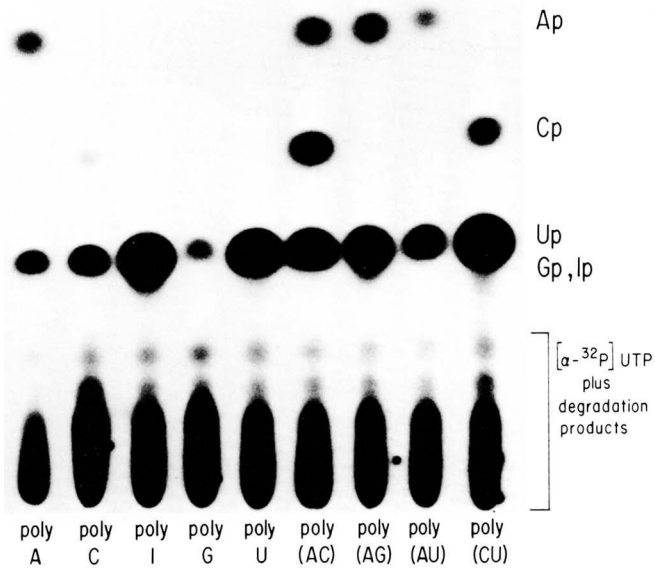


FIG. 4. **Nearest neighbor analysis of terminal uridylyl transferase reaction products.** Different RNA homo- and heteropolymers were substituted for oligo(U) in the terminal uridylyl transferase reaction and the products were subjected to a nearest neighbor analysis (see under "Experimental Procedures"). The figure shows an autoradiogram of the chromatography plate. The material at the bottom consists of degradation products of the unincorporated label.

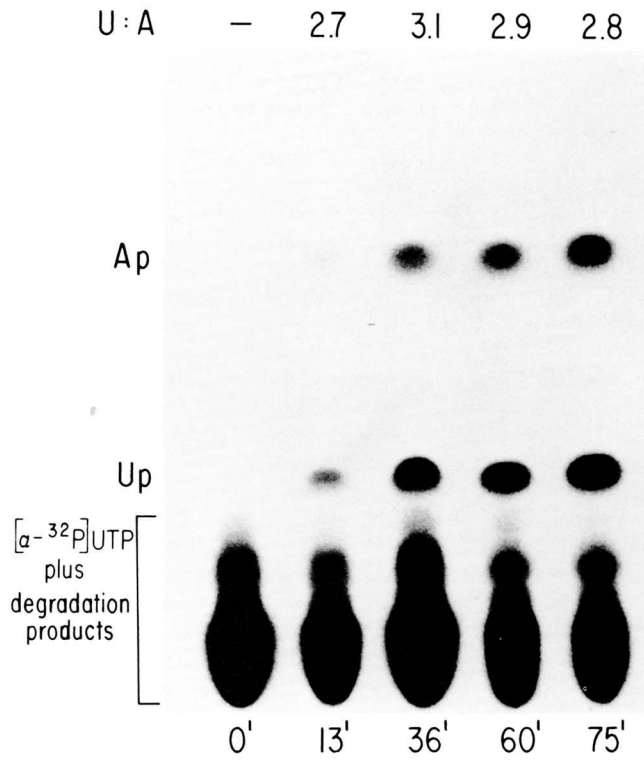


FIG. 5. **Nearest neighbor analysis of terminal uridylyl transferase products made from poly(A) primer at different times of incubation.** This experiment is very similar to that shown in Fig. 4. Here the primer was in all cases poly(A), and terminal uridylyl transferase reaction mixtures were incubated for varying lengths of time. The exposure was not made on preflashed film, and is not linear. Spots were removed for measurement of radiactivity as described under "Experimental Procedures," and ratios of radioactivity in Up and Ap spots are shown.

polymers tested (poly(A), poly(C), poly(I), poly(G), poly(U), poly(A,C), poly(A,G), poly(A,U), and poly(C,U)) could serve as good acceptors, with the exception of poly(G), which served poorly. Poly(A,G) could be labeled on G ends as well as A ends, implying that the problem with poly(G) was in the homopolymer structure, rather than G ends *per se*. All lanes showed more radioactivity in the Up spot than in other Np spots. We therefore conclude that, on average, those molecules which have had U's added to their 3′ ends will have more than two U's added. The number of U's added appears to depend on the nature of the substrate because the ratio of Up to Np radioactivity varied from substrate to substrate. In the case of poly(C), for example, it was about 18:1, indicating that an average of about 19 U's are added to the end of each molecule of poly(C) which receives U's. In contrast, poly(A) accepted four U's per molecule, on average, before the uridylyl transferase stopped. In a time course using poly(A) as a primer, the ratio of radioactivity in Up spots to Ap spots was about 3.0 at all times, suggesting that, on average, molecules with U tails have only four U's added (Fig. 5). Oligo(U) molecules receive long tails of heterogeneous lengths, averaging 30 to 40 nucleotides, increasing in average length with time, and extending to over 100 nucleotides (Fig. 6). The long tails on oligo(U) and poly(C) contrast to the short tails on poly(A), suggesting that bonding of the newly formed oligo(U) tail to the primer may abort elongation. The ratios between Up spots and other Np spots seen in Fig. 4 are consistent with this explanation. Zabel and co-workers see a similar effect of primer specificity in studies on a plant terminal uridylyl transferase.[4]

[4] P. Zabel, personal communication.

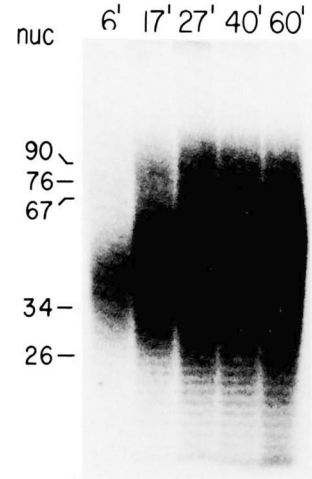


FIG. 6. **Products of terminal uridylyl transferase reaction.** In this experiment standard terminal uridylyl transferase reaction mixtures were incubated for varying lengths of time, and the products were analyzed on 15% urea/polyacrylamide gels. The label was [α-$^{32}$P]UTP, and the RNA primer was oligo(U). The nucleotide lengths of several markers are shown.

Because the terminal uridylyl transferase activity was originally identified as a contaminant in eIF-2 fraction purified from ribosomes, we wanted to know if it was associated exclusively with ribosomes. For convenience, we chose to use HeLa cells in this experiment, instead of rabbit reticulocytes. A crude HeLa cell extract was cleared of nuclei, and fraction-

ated on a 15 to 30% sucrose gradient. Activity was measured across the gradient fractions using the terminal uridylyl transferase assay. Ribosomal RNAs and poliovirions were used as markers. In each of several experiments the terminal uridylyl transferase activity co-sedimented with 40 and 80 S ribosomal particles. A representative experiment is shown in Fig. 7. In most, but not all experiments, there was also a small peak of activity at about 160 S. Some activity was found in the top fraction with most of the cytoplasmic protein. To determine if the lower level of activity in the top fraction was due to inhibitors in that fraction, we mixed active fractions with the top fraction and assayed the mixtures. This caused a 30 to 40% inhibition in both the 40 and 80 S peak fractions. Furthermore, the activity in the top fraction activated upon dilution. Thus, there may be a significant amount of activity free in the cytoplasm. Nevertheless, it seems clear that there is some association of the terminal uridylyl transferase with ribosomes and the small ribosomal subunit.

*Terminal Uridylyl Transferase Activity Is Probably the Host Factor Activity in Step VI eIF-2*—Although it is evident that step VI eIF-2 contains both terminal uridylyl transferase and host factor activities, nothing described yet implies that the terminal uridylyl transferase enzyme is host factor. The eIF-2 preparation contains only a few proteins other than eIF-2 (Fig. 1) but it could still be coincidental that terminal uridylyl transferase and host factor have purified together. To examine this question, terminal uridylyl transferase activity, eIF-2 concentration, and replicase stimulating activity were assayed across the glycerol gradient used for the final step in step VI eIF-2 purification (Fig. 8). Fraction 1 was the top of the gradient. Only fractions containing eIF-2 or appreciable levels of uridylyl transferase and/or host factor-like activity are shown in the figure. Fractions 14 to 16 and fractions 17 to 19 were pooled and assayed for both activities. Fractions 14 to 16 contained a small amount of both activities, fractions 17 to 19 contained almost none. The uridylyl transferase and host factor-like activities appeared to be coincident, and the peaks of those activities were about one fraction away from the peak eIF-2 concentration. This result is consistent with the idea that the same enzyme is responsible for both RNA polymerization-related activities. More rigorous examination of this question is clearly required.

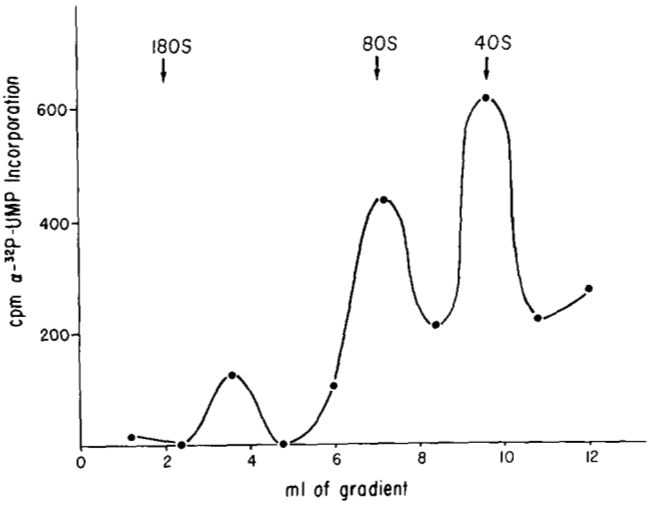


FIG. 7. **Distribution of terminal uridylyl transferase activity in HeLa cell cytoplasm.** HeLa cell cytoplasm was prepared and fractionated as described under "Experimental Procedures." Gradient fractions were assayed for terminal uridyl transferase activity using the standard assay. RNA samples from each fraction were prepared by phenol extraction and ethanol precipitation and analyzed on agarose gels to determine the sedimentation positions of ribosomal subunits (and 180 S poliovirions in a parallel gradient).

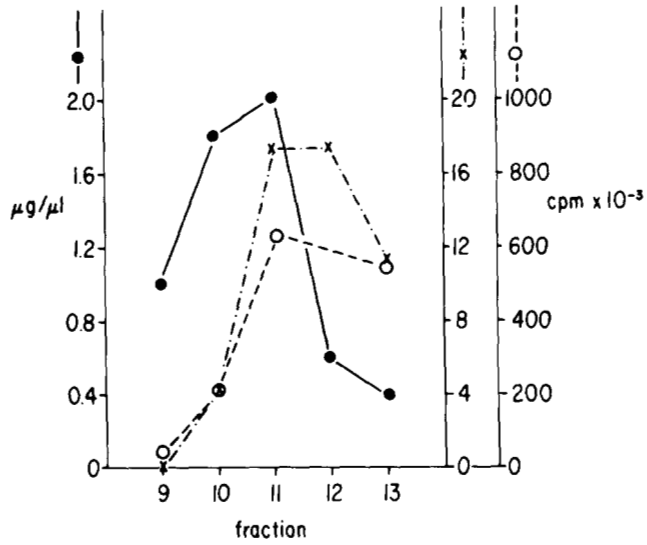


FIG. 8. **eIF-2 concentration, terminal uridylyl transferase activity, and replicase-stimulating activity across a glycerol gradient used late in the purification of eIF-2.** Fractions from a glycerol gradient used in the purification of eIF-2 were analyzed for eIF-2 concentration (●), terminal uridylyl transferase activity (–·×·–), and host factor-like activity (–○–). Enzymatic assays are described under "Experimental Procedures." Only fractions with eIF-2 or activity peaks are shown. Fraction 12 was not available for assay of activity in the replicase reaction.

## DISCUSSION

Initiation of RNA synthesis *in vitro* by the poliovirus replication system has an absolute requirement for either host factor or a pre-formed oligo(U) primer (9–17). While investigating sources of host factor activity we found it associated with partially purified eIF-2. In concentrated initiation factor preparations, we find a terminal uridylyl transferase activity. Its presence is evident when almost any RNA is used as primer. The RNA is elongated by addition of UMP residues in a $Mg^{2+}$-dependent reaction. When the primer is poly(A), an average of 4 UMP residues are added and then the reaction ceases. In all probability, the double-stranded product formed by snap-back of oligo(U) on the poly(A) primer prevents further elongation This provides a model for initiation of poliovirus replication that will be detailed after certain points in the data are discussed.

At present, we can only say that an active terminal uridylyl transferase is found in the eIF-2 fractions which serve as host factor but lacking from eIF-2 fractions without host factor activity. The terminal uridylyl transferase activity in the preparation co-sediments with host factor activity. The most pure fractions containing terminal uridylyl transferase activity have two proteins not present in completely pure eIF-2; one or both of the 60,000 and 95,000 molecular weight proteins could be the host factor. Previously host factor was thought to be a 67,000 molecular weight protein in HeLa cells, but that identification was not absolutely certain (10, 12). The activity studied here was purified from rabbit reticulocytes and may therefore have different physical properties. The terminal uridylyl transferase activity sediments more rapidly than eIF-2, but has not yet been separated from eIF-2, suggesting that it may be bound to it. Otherwise, it must be part of a complex which associates with ribosomes and sediments somewhat ahead of the approximately 150,000 molecular

weight eIF-2 complex. HeLa host factor showed a partial association with ribosomes (9, 10, 12), consistent with our data.

There is at present no way to be certain that the terminal uridylyl transferase activity and host factor are the same protein. It is also possible that host factor, when purified by its replicase-stimulating activity, is not a terminal uridylyl transferase but that terminal uridylyl transferase can show host factor activity. These questions are presently being investigated.

Perhaps the strongest evidence that the previously characterized HeLa host factor might be a terminal uridylyl transferase comes from the identification of double-length reaction products in the poliovirus *in vitro* replicase reaction using that protein. These have been described by Young *et al.* (56) who have also detected low levels of double-length products *in vivo*.

Although terminal uridylyl transferase may not be the only protein that acts as host factor and may not participate in viral replication in the cell, our *in vitro* results suggest a model for replication initiated by terminal uridylyl transferase (Fig. 9). This model, which is similar to the model proposed by Young *et al.* (56), can only be applicable to the synthesis of minus strands on a plus strand template, and not to the synthesis of plus strands on a minus strand template. We postulate that terminal uridylyl transferase adds a short stretch of U's onto the poly(A) end of a poliovirion RNA molecule. This oligo(U) tail will, as stated above, soon hydrogen bond to the poly(A) and elongation will stop. The poliovirus polymerase, however, is known to use just such oligo(U)-poly(A) complexes as primer templates. When pre-formed oligo(U) polio RNA is used, the oligo(U) primes synthesis of a full length product (14, 15). In the present model, employing a terminal uridylyl transferase as host factor, the product would be a double-stranded RNA structure with a loop connecting the 3′ end of the template with the 5′ end of the product. This might serve as a substrate for a specific nuclease which could make a cut at the poly(A)-poly(U) border liberating two strands. In particular, we speculate that the covalent dimer might be positioned on cellular membranes, and this nicking might be performed by a membrane-associated precursor to VPg, which simultaneously makes the cleavage and forms the RNA-protein bond. There are precedents for DNA nicking-closing enzymes which go through a stable DNA-protein linkage via a phosphate-tyrosine linkage (39–43). The linkage between polio RNA and VPg is also a phosphate-tyrosine bond (2, 44). There are also precedents for replication-priming with a 5′-terminal-linked protein, in which a protein-nucleotide complex serves in initiation (45–49), but in such cases the nucleic acid-protein bond has invariably involved serine, rather than tyrosine (48, 49). In our model for VPg attachment, later or coincident processing might cleave VPg from its protein precursor, perhaps helping to make the reaction irreversible.

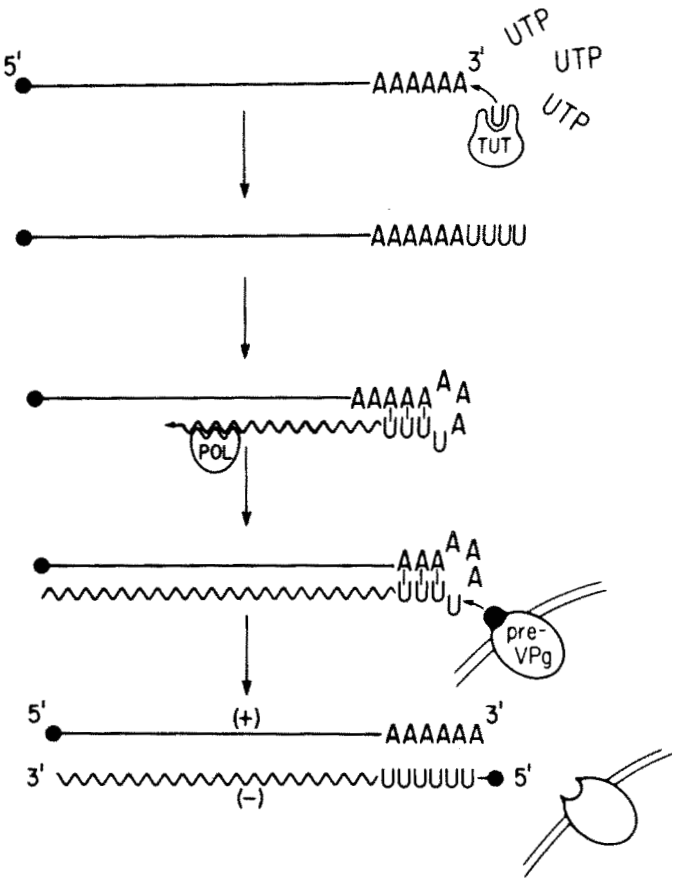


FIG. 9. **Model for the role of terminal uridylyl transferase in the replication of poliovirus RNA.** *TUT*, terminal uridylyl transferase; *POL*, viral polymerase; *pre-VPg*, a protein precursor to genome-linked peptide VPg; and the *dark circle*, VPg. Poly(A) and poly(U) tracts are not shown in their entirety, and the figure is not drawn to scale. *Straight* and *wavy lines* represent positive and negative sense strands, respectively.

Although there is no direct evidence for this model, several facts make it worth considering, in light of our *in vitro* results. Encephalomyocarditis virus, another picornavirus, has been shown to produce palindromic dimer forms *in vivo* (38). As would be predicted from the oligo(U) addition/fold back priming model, poly(A) has been shown to be necessary for the infectivity of poliovirus and other picornaviruses (5, 50). Precursors to VPg have been found localized in smooth membranes (51, 52). The crude polio replicase complex associates spontaneously with smooth membranes *in vitro* (53, 54), although the polymerase active site is probably not intimately associated with the membrane (54). VPg-pUpU has been isolated from infected cells (21), and it is formed in *in vitro* membrane fractions (22). This molecule might result from premature cleavage of a fold back structure. The *in vitro* conditions for production of VPg-pUpU differ from those for the synthesis of longer RNAs in the same crude membrane system (22).

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SUPPLEMENTARY MATERIAL TO:
POLIOVIRUS REPLICASE STIMULATION BY TERMINAL URIDYLYL TRANSFERASE
Nancy C. Andrews, Daniel Levin and David Baltimore

## EXPERIMENTAL PROCEDURES

### Radiochemicals

[$\alpha$-$^{32}$P]UTP, -CTP, -GTP and -ATP (about 760 Ci/mMol) were purchased from New England Nuclear. [$\gamma$-$^{32}$P]ATP (about 700 Ci/mMol) was purchased from I.C.N. [$^{32}$P] orthophosphate was purchased from New England Nuclear, acid free.

### Preparation of Virion RNA and Viral RNA Polymerase

Polio virion RNA was prepared as described by Baron and Baltimore (31). [$^{32}$P]-labeled polio RNA was prepared essentially as described by Hewlett et al.(32), except polyvinyl sulfate precipitations and oligo(dT)-cellulose chromatography were omitted.

Viral RNA polymerase was prepared by two methods. Protocol 1, used to prepare "partially purified" polymerase, was according to Baron and Baltimore (10). This protocol involves passing an extract from polio-infected cells through phosphocellulose and poly(U)-Sepharose columns, and results in a partially purified enzyme which is relatively free of host factor activity. The amount of residual host factor activity varies from preparation to preparation. Protocol 2 resulted in a much more highly purified polymerase. This method is a combination of the purification protocols of Baron and Baltimore (10) and Flanegan and Van Dyke (14), with modifications. Three liters of HeLa cells were grown in spinner flasks, infected and harvested as described (10). Fifteen ml of 10 mM Tris-HCl pH 8.0, 10 mM NaCl, plus a mixture of protease inhibitor peptides (Boehringer-Mannheim) were added to the cell pellet. All subsequent steps were carried out at 0 to 4 C. The cells were allowed to swell for ten minutes, and then disrupted in a dounce homogenizer. Nuclei were removed by a 700 x g centrifugation. The cytoplasmic supernatant was centrifuged for 90 min at 200,000 x g. The pellet was discarded, and 3.55 g of ammonium sulfate was added to 17 ml of high speed supernatant, resulting in 35% saturation. The precipitate was collected by centrifugation for 10 minutes at 10,000 x g and redissolved in 15 ml of Buffer II (50 mM Tris-HCl pH 8.0, 20% v/v glycerol, 0.5% Nonidet P-40, 5 mM $\beta$-mercaptoethanol). The redissolved precipitate was loaded onto a phosphocellulose column (Whatman P11) which had been prepared according to Burgess (33), and pre-equilibrated with Buffer II. After loading and washing, the column was developed with a 200 ml, 0 to 1 M KCl gradient in Buffer II. Fractions were assayed using the poly(U) polymerase assay described by Baron and Baltimore (10), and peak fractions (eluting at about 150-200 mM KCl) were pooled. The phosphocellulose material was diluted to approximately 50 mM KCl final with Buffer II, supplemented with protease inhibitor peptides, and loaded onto a DEAE-Sephacel column (pre-equilibrated with Buffer II/50 mM KCl) at a rate of 1 ml/minute. This column was washed with Buffer II/50 mM KCl and developed with a 50-500 mM KCl gradient in Buffer II. The peak fractions (again at about 150-200 mM KCl) were pooled and dialyzed overnight versus Buffer II. This material was loaded very slowly onto a poly(U) Sepharose column which had been pre-equilibrated with Buffer II/50 mM KCl/0.02% Brij 35. A gradient of 50-500 mM KCl in Buffer II/0.02% Brij 35 was used to develop the column. The peak fractions were pooled and dialyzed against solid sucrose overnight, in the cold. This dialysate was further dialyzed against Buffer II/0.02% Brij 35, aliquoted, and stored at -70 C. This preparation contained only 2 major proteins (apparently polio proteins NCVP2 and p63) when analyzed by electrophoresis through an SDS/polyacrylamide gel followed by silver staining .

### Replicase and Poly(U) Polymerase Reactions

Replicase reactions were carried out as described by Baron and Baltimore (10), except that dithiothreitol was used in place of dithioerythritol, and 2 Ci [$\alpha$-$^{32}$P] UTP plus 2 $\mu$M unlabeled UTP (or other [$\alpha$-$^{32}$P]NTP plus unlabeled NTP) were used instead of [$^3$H]CTP. Unlabeled NTPs were present at 200 $\mu$M. Between 0.2 and 1 $\mu$l of eIF2 fraction (about 0.1 - 0.6 $\mu$g total protein) replaced host factor and oligo(U). Reactions were normally incubated for 60 minutes.

Poly(U) polymerase reactions were also carried out as described by Baron and Baltimore (10), except [$^{32}$P]UTP was used in place of [$^3$H]UTP.

### Terminal Uridylyl Transferase Assay

The terminal uridylyl transferase assay was performed in a reaction volume of 50 $\mu$l, with 40 mM HEPES-KOH pH 8.0, 8 mM Mg acetate, 30 $\mu$M UTP, 2.5 $\mu$g/ml oligo $(U)_{10-20}$ (Collaborative Research), 4 mM dithiothreitol, 0.5 to 5 $\mu$Ci/reaction [$\alpha$-$^{32}$P]UTP. From 0.1 to 2 $\mu$l of the enzyme fraction was added per reaction, and the mixtures were incubated for 30 to 60 minutes at 30 C. After incubation, a portion of the reaction was spotted onto DE81 paper (Whatman), and papers were washed thoroughly with 5% $Na_2PO_4$-$7H_2O$. The washed papers were dehydrated with an ethanol wash and an ether wash, allowed to dry, and counted in Bray's scintillant (New England Nuclear).

### Nearest Neighbor Analysis

The reaction was similar to the terminal uridylyl transferase assay, except polyribonucleotides were substituted for oligo(U). Each reaction received 0.5 $\mu$l of enzyme, and was incubated for 60 minutes. At the end of the incubation 5 $\mu$l of each sample was spotted onto DE81 paper and processed as described above. Carrier tRNA (15 g), $NH_4$ acetate to 2 M, and 2.5 volumes of ethanol were added to precipitate the remainder of each sample. The 2 M $NH_4$ acetate/ 2.5 volume ethanol procedure was repeated two more times after the precipitate was redissolved in $H_2O$. Finally the pellets were washed with cold 70% ethanol and redissolved in 9 $\mu$l of 20 mM $NH_4$ acetate pH 4.5 (the precipitations and washes removed over 99% of the unincorporated label). One unit $T_2$ RNase (Calbiochem) was added, and, after a 60 minute incubation at 50 C, the samples were dried by lyophilization and redissolved in 3 $\mu$l $H_2O$. Samples were spotted onto thin layer cellulose chromatography plates (Merck) and developed with an isobutyric acid/$NH_4OH$/$H_2O$ (66:1:33) solvent overnight, as described by Silberklang et al. (34). The plates were then dried, and used to expose Kodak XAR-5 film with the aid of an intensifying screen. Unlabeled markers (at a concentration of about 12.5 mM) were spotted in parallel, and could be visualized by transilluminating the plate with UV light. Spots were lifted for scintillation counting by painting them with 10% nitrocellulose in ethanol:acetone (1:1), allowing the coating to dry, and peeling off the cellulose plaques (34). The plaques were then redissolved in Bray's scintillant and counted.

### Extension of 5' End-Labeled Oligo(U)

This experiment is similar to an experiment described by Zabel et al.(30). Kinased $oligo(U)_{19}$ was prepared by the following method. About 45 $\mu$g of $oligo(U)_{10-20}$ (Collaborative Research) was treated with calf intestinal phosphatase (Boehringer-Mannheim) in 50 mM Tris-HCl pH 8.0. After a 30 minute incubation at 37 C, the mixture was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and precipitated with 0.3 M Na acetate plus 2.5 volumes of ethanol. The material was redissolved in $H_2O$, and adjusted to 50 mM Tris-HCl pH 7.5, 5 mM Mg acetate, 150 $\mu$Ci/25 l [$\gamma$-$^{32}$P]ATP, 0.5 $\mu$l/25 $\mu$l reaction T4 polynucleotide kinase (Boehringer-Mannheim), and incubated at 37 C, for 30 minutes. An equal volume of 7 M urea, 25% sucrose, 1X TBE, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol was added (1X TBE is 0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA). The samples were electrophoresed through preparative urea/TBE/15% polyacrylamide gel (35). After electrophoresis, the gel was used to expose Kodak XAR-5 film for 1-2 minutes. A gel band was excised, rinsed briefly with $H_2O$ to remove some urea, and then soaked in a small volume of 40 mM Tris acetate pH 8.2, 20 mM Na acetate, 1 mM EDTA for 2 hours at 37 C to elute the RNA. The supernatant was then withdrawn from the gel slice, adjusted to 0.3 M Na acetate, and 2.5 volumes of ethanol were added to precipitate the RNA. The precipitate was washed several times with cold 70% ethanol, and redissolved in $H_2O$. This preparation was aliquoted and stored at -20 C.

The kinased oligo(U) prepared in this way was added to a terminal uridylyl transferase assay. No unlabeled oligo(U) was added; unlabeled UTP replaced [$\alpha$-$^{32}$P]UTP. Portions were taken from the reaction at various times of incubation, mixed with an equal volume of the urea/dye mixture, and kept on dry ice until all samples were ready. Just before loading a urea/TBE/12% polyacrylamide gel (35) the samples were heated to 70 C for 3 minutes. Non-fractionated, kinased oligo(U) was used as markers. After electrophoresis the gel was transferred to a plastic backing and used to expose film, with an *intensifying screen*.

### Protein Gels

SDS-polyacrylamide protein gels were prepared and run essentially as described by Baron and Baltimore (10), and silver stained (36) omitting the glutaraldehyde fixation step[5].

### Sucrose Gradient of HeLa Cytoplasm

Two liters of HeLa cells growing in suspension at 4 x $10^5$/ ml were harvested, washed twice with PBS, and allowed to swell on ice in 10 mM HEPES-KOH pH 8.0, 15 mM NaCl, 1.5 mM $MgCl_2$, 5 mM $\beta$-mercaptoethanol for 10 minutes. They were homogenized in a Dounce homogenizer and centrifuged at 27,000 x g, 4 C, for 30 minutes to remove nuclei. Portions (0.5 ml) of the resulting supernatant were loaded onto 12 ml 15-30% sucrose gradients in the same buffer as used for lysis. The gradients were centrifuged at 40K rpm in a Beckman SW 41 rotor for 3 hours at 4 C. The gradients were collected from the bottom of the tube. Five $\mu$l portions of each fraction were used as the enzyme in the terminal uridylyl transferase assay described above, with 5 $\mu$g/ml actinomycin D added. Other portions of each fraction were either extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and precipitated with ethanol to be analyzed on RNA gels; diluted to measure $OD_{260}$; or assayed for relative protein content using the Bradford reagent (37) in the BioRad assay. In two experiments, polio virions were used to obtain a 180 S marker.

### Purification of eIF2 and Glycerol Gradient

The eIF2 preparation containing uridylyl transferase activity was purified from a ribosomal salt wash of rabbit reticulocyte lysate. Reticulocyte ribosomes were obtained from lysate by sedimentation overnight through a 50% glycerol cushion in 10 mM Tris-HCl pH 7.7/ 2 mM $MgCl_2$/ 25 mM KCl. The ribosome pellet was resuspended by gentle homogenization in 25 mM Tris-HCl pH 7.7/ 80 mM KCl/ 1 mM DTT/ 0.2 mM EDTA/ 10 % glycerol (buffer A). Ribosome salt wash was prepared at 0 C by extraction of a ribosome suspension for 10 min with 0.5 M KCl in 10 mM Tris-HCl (pH7.7)/2 mM Mg acetate; after centrifugation, the supernatant was concentrated by precipitation in $(NH_4)_2SO_4$ (0-60%) and resuspended in buffer A. The extract was chromatographed on DEAE-cellulose (Whatman DE-52); eIF2 was obtained by stepwise elution at 0.1 M- 0.2 M KCl in buffer A. After concentration as before, the eIF2 fraction was chromatographed on phosphocellulose (Whatman P11), and was eluted by stepwise elution at 0.4 - 0.6 M KCl in buffer A. After concentration as before, the eIF2 fraction was centrifuged at 2 C in 15-50% linear glycerol gradients in buffer A, at 110,000 x g for 67 h. Fractions were collected by upward displacement. eIF2 sedimented as a broad peak with fraction purities of 50-80%, and relative retardation rates of 0.50 for peak eIF2 and 0.55 for peak uridylyl transferase (assayed as described elsewhere under "Experimental Procedures"). Re-centrifugation of peak eIF2 fractions in similar gradients yielded eIF2 purities of < 95%. Step VI eIF2 refers to eIF2 purified through the glycerol gradient sedimentation step; step VI-N eIF2 refers to a preparation which was purified through the same steps, but more narrow cuts were taken at peak fractions and the step VI material was re-centrifuged through a second glycerol gradient. This resulted in nearly homogeneous eIF2.

5. Richard Carthew, unpublished modification.